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Title: Biofilms formed by isolates from recurrent vulvovaginal candidiasis patients are heterogeneous and insensitive to fluconazole

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26 **Abstract (75 word limit)**

27 Vulvovaginal candidiasis (VVC) is a global health problem affecting ~75% of
28 women at least once in their lifetime. Here we examined the epidemiology of
29 VVC from a patient cohort to identify the causative organisms associated with
30 VVC. Biofilm forming capacity and antifungal sensitivity profiles were also
31 assessed. We report a shifting prevalence of *Candida* species with
32 heterogeneous biofilm forming capacity, both of which are associated with
33 altered antifungal drug sensitivity.

34 Fungal infections play a surprisingly substantial, yet unrecognised, health
35 burden on the global population (1). Vulvovaginal candidiasis (VVC) is one
36 example of these, where it is estimated to be the most common fungal infection
37 in a number of countries worldwide (2-4). Approximately 138 million women
38 worldwide complain of >4 episodes of VVC per year due to treatment failure,
39 clinically defined as recurrent VVC (RVVC) (5-7). These unresolved infections
40 not only have a high impact on the quality of life of these women, but can also
41 lead to further health complications (8). *Candida albicans* is historically
42 reported as the predominant organism isolated from VVC, accounting for over
43 90% of infections (9, 10). However, evidence of a dynamic shift in yeast
44 epidemiology has been demonstrated through an increasing prevalence of
45 non-*C. albicans* species (NCAS), which accounts for 11-80% of infections,
46 depending on geographical location (11). Nevertheless, *C. albicans*, a well-
47 characterised biofilm-forming organism, remains a prominent pathogen in this
48 disease. Resistance to antifungal therapy as a result of biofilm formation is a
49 likely contributor to failed treatment. While it is widely accepted that biofilms
50 contribute to the pathogenesis of bacterial vaginosis (BV) (12, 13), their role in
51 VVC remains contested despite the overwhelming evidence to suggest
52 otherwise (14-16).

53 An anonymised series of high vaginal swabs (HVS, [n=300]) obtained from
54 women attending GP and referral clinics in the NHS Greater Glasgow & Clyde
55 area, for at least the second time throughout April 2016 (17). These women
56 were symptomatic at the time of sampling, with the causative organism
57 identified using matrix-assisted laser desorption/ionisation-time of flight

(MALDI-TOF), with *Escherichia coli* used pre and post yeast sampling to ensure accuracy of testing.

Seventy one percent (n=212) identified as *C. albicans*, followed by 15% (n=47) *C. glabrata*, 6% (n=17) *C. dubliniensis*, 3% (n=10) *C. parapsilosis* (Figure 1). The remaining 5% of isolates included *C. tropicalis*, *C. lusitaniae* and *C. guilliermondii*. These data are line with recent epidemiological patterns showing a shift in NCAS within VVC (11). However, a caveat of our study is the limitation of a single geographical location, which may influence the species distribution. Future studies should include various institutes globally in order to fully assess the shift in VVC epidemiology.

To determine the biofilm forming capability of these isolates, all VVC strains (n=300) were standardised to 1×10^6 cells/mL in RPMI-1640 and grown as biofilms in 96 well plates for 24 h. Biofilms were washed with PBS and biomass assessed using the crystal violet (cv) assay (18). Here we have shown that vaginal isolates were able to form differential biofilms, regardless of species (Figure 2). *C. albicans* displayed the greatest heterogeneity with regards to biofilm biomass, with isolates ranging from OD_{570nm} 0.008 to 1.478, with a mean of 0.416. The second most prevalent species, *C. glabrata*, had significantly lower biomass than *C. albicans* (p<0.05) and *C. dubliniensis* (p<0.01), with a mean OD_{570nm} 0.271. This apparent biofilm heterogeneity may contribute to the management of VVC infections, as these communities are known to be notoriously recalcitrant to antifungal therapy, and biofilm heterogeneity has been shown to correlate with *in vitro* antifungal therapy (18).

82 Planktonic and biofilm antifungal susceptibility testing was carried out as
83 described previously to determine the minimum inhibitory concentrations
84 (MICs) (19). Briefly, cells were standardised in RPMI-1640 before being treated
85 with fluconazole (FLZ) (Sigma, Dorset, UK) for 24 h, at a range of
86 concentrations (0.0625 to 32 mg/L). Planktonic MIC's (pMIC) were determined
87 as the lowest concentration able to completely inhibit growth visually. Sessile
88 MIC's (sMIC) were performed on 24 h preformed biofilms, with sMIC recorded
89 at 50% inhibition using an XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-
90 tetrazolium-5-carboxanilide) metabolic reduction assay (20). Here we have
91 shown FLZ, the first line antifungal used to treat VVC, was ineffective against
92 most isolates, with planktonic MIC's ranging from <0.0625 to >32 mg/L (Table
93 1). Specifically, the pMIC₅₀ for FLZ was 4 mg/L, for *C. albicans*, *C. glabrata* and
94 *C. dubliniensis*, though for biofilms this was >32 mg/L. When planktonic cells
95 were stratified based on *C. albicans* and NCAS it was shown that 41% and
96 26% of the isolates were insensitive to FLZ at >32 mg/L, respectively. Whereas
97 for sessile cells, this rose to 51% and 56% of the isolates, respectively.
98 Interestingly, similar susceptibility profiles were observed for *C. albicans* and *C.*
99 *glabrata*, despite *C. glabrata* known to be a low biofilm former (21). This
100 reduced sensitivity in *C. glabrata* can be associated with its intrinsic resistance
101 to fluconazole, due to the overexpression of multidrug transporters (22).

102 VVC is not a reportable disease, making epidemiological studies difficult.
103 However, this study provides a snapshot of the species identified within a VVC
104 population, demonstrating that NCAS are responsible for an increasing number
105 of these infections. This corresponds with previous studies reporting an on-
106 going dynamic shift in yeast epidemiology (23, 24), potentially driven by

107 inappropriate use of over-the-counter azoles (10). Irrespective, *C. albicans*
108 remained the most dominant species in this study, which questions why a high
109 number of isolates displayed reduced susceptibility to FLZ. We demonstrated
110 the ability of these clinical isolates to form heterogeneous biofilms, and the
111 presence of these communities in VVC may explain why *C. albicans* infections
112 remain unresponsive to FLZ therapy; an antifungal highly ineffective against *C.*
113 *albicans* biofilms (25). We cannot discount the potential for heteroresistance
114 phenotypes within these populations (26). The contribution of biofilms to VVC
115 pathogenesis remains poorly understood, though many researchers are
116 beginning to consider them important determinants of disease (14, 15), further
117 emphasising the need for research in this field. Collectively, the data from this
118 investigation highlights the necessity for careful consideration of the causative
119 organism in VVC, the biofilm phenotype and its accentuated antifungal
120 sensitivity profiles, all of which may improve antifungal treatment in this area.

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Figure 1: Distribution of organism isolated from VVC patients. Three hundred VVC isolates were identified using MALDI-TOF, with yeast species proportionally represented.

Figure 2: VVC isolates display varied biofilm formation. Three hundred VVC isolates were screened for biofilm formation using a biomass stain, as described in the methods. Each isolate was tested in quadruplicate, with the mean represented. Statistical analysis was carried out using a one-way ANOVA (* $p < 0.05$, ** $p < 0.01$).

212 **Table 1: Susceptibility profile of *Candida* vaginal isolates to fluconazole**

213

Fluconazole Minimum Inhibitory Concentration (mg/L)										
n = 300	<i>C. albicans</i> (n=212)		<i>C. glabrata</i> (n=47)		<i>C. dubliniensis</i> (n=17)		<i>C. parapsilosis</i> (n=10)		Others (n=14)	
	PMIC*	SMIC**	PMIC	SMIC	PMIC	SMIC	PMIC	SMIC	PMIC	SMIC
Range	0.0625 – >32	0.125 - >32	<0.0625 – >32	0.5 - >32	0.125 – >32	0.125 - >32	1 - >32	1 - >32	0.0625 – >32	1 - >32
MIC₅₀	4	>32	4	>32	4	>32	1	4	1	>32
MIC₉₀	>32	>32	>32	>32	>32	>32	16	>32	>32	>32

214

215 *PMIC – Planktonic minimum inhibitory concentration, **SMIC – sessile minimum inhibitory concentration

Table 1: Susceptibility profile of *Candida* vaginal isolates to fluconazole

Fluconazole Minimum Inhibitory Concentration (mg/L)										
n = 300	<i>C. albicans</i> (n=212)		<i>C. glabrata</i> (n=47)		<i>C. dubliniensis</i> (n=17)		<i>C. parapsilosis</i> (n=10)		Others (n=14)	
	PMIC*	SMIC**	PMIC	SMIC	PMIC	SMIC	PMIC	SMIC	PMIC	SMIC
Range	0.0625 – >32	0.125 - >32	<0.0625 – >32	0.5 - >32	0.125 – >32	0.125 - >32	1 - >32	1 - >32	0.0625 – >32	1 - >32
MIC₅₀	4	>32	4	>32	4	>32	1	4	1	>32
MIC₉₀	>32	>32	>32	>32	>32	>32	16	>32	>32	>32

*PMIC – Planktonic minimum inhibitory concentration, **SMIC – sessile minimum inhibitory concentration

Figure 1

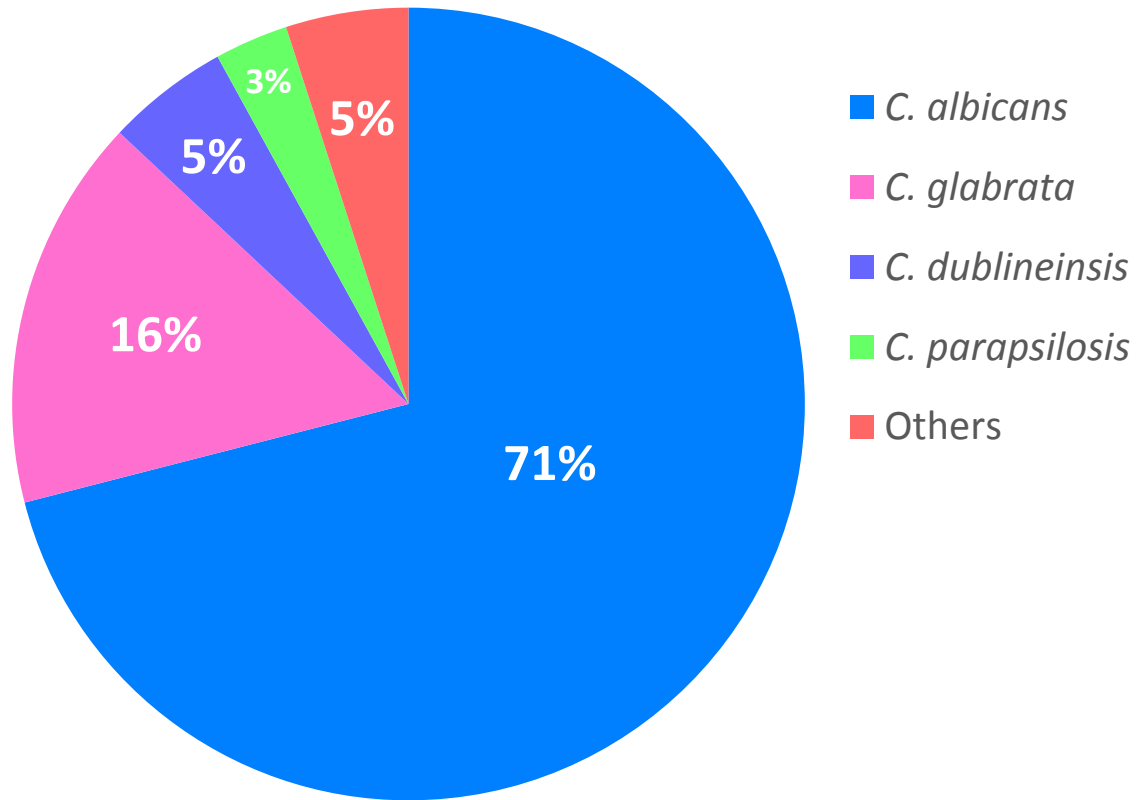


Figure 2

